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(54) Title: ANTIFUNGAL ANTIBIOTIC CEPACIDINE A		
(57) Abstract <p>Novel antifungal antibiotics Cepacidine A (A₁ and A₂), a novel microorganism <i>Pseudomonas cepacia</i> AF 2001 producing the same, and a process for producing the said antibiotics are disclosed.</p>		

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ANTIFUNGAL ANTIBIOTIC CEPACIDINE A

BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention relates to novel antifungal antibiotics Cepacidine A(A₁ and A₂), a novel microorganism *Pseudomonas cepacia* AF 2001 producing the same, and
10 a process for producing the said antibiotics.

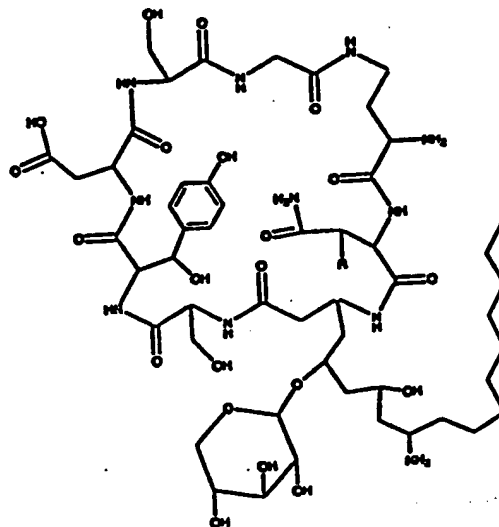
Description of the Prior Art

As a variety of antifungal agents such as griseofulvin,
15 nystatin, amphotericin-B and the like were separated from actinomycetes, chemotherapies for the treatment of fungal infections have been established. Another group of antifungal agents such as blasticidin, kasugamycin, polyoxin and validamycin was separated from actinomycetes
20 and has been utilized in controlling fungal infections of plants. Thereafter, many antifungal substances were not only separated from various microorganisms occurring in nature but also produced by synthetic, chemical procedures. However, due to their poor efficacy or
25 toxicity, most antifungal agents have not been utilized in commerce.

Antifungal agents in practical use do not function in a broad spectrum of activities nor do they exhibit a
30 safe level of toxicity. These problems make it difficult to remedy deep antifungal infections. Moreover, since these antifungal agents do not have enough activities to kill fungi completely, rather having fungistatic activities, the use of those antifungal agents requires a
35 long period of therapy. Therefore, there exists a need to develop novel antifungal agents having low toxicity, quick efficacy and fungicidal activity.

01 SUMMARY OF THE INVENTION

Cultivation of the novel microorganism *Pseudomonas cepacia* AF 2001 yields novel antibiotic substances Cepacidine A
05 having powerful activities against various yeasts and fungi, which have the formula:



20 wherein R is H or OH. Where R is H, Cepacidine A₂ was designated; and where R is OH, Cepacidine A₁ was designated.

25 DESCRIPTION OF THE DRAWINGS

Fig.1 shows the ultraviolet spectrum of Cepacidine A.

Fig.2 shows the infrared spectrum of Cepacidine A in potassium bromide.

30 Fig.3 shows the fast atom bombardment mass spectrum of Cepacidine A.

Fig.4 shows the 400 MHz ¹H NMR spectrum of Cepacidine A in deuterated dimethylsulfoxide.

35 Fig.5 shows 100 MHz ¹³C NMR spectrum of Cepacidine A in deuterated dimethylsulfoxide.

Fig.6 shows the pyrolyzed gas chromatograms of Cepacidine A, asparagine and aspartic acid.

01 DETAILED DESCRIPTION OF THE INVENTION

The Microorganism

05 *Pseudomonas cepacia* AF 2001 producing novel antifungal
 Cepacidine A was isolated from the soil samples collected
 in Munchon, Kyunggi-Do, Korea. The strain AF2001 was
 deposited at the Korean Federation of Culture Collections,
 Seoul, Korea, under the accession number KFCC 10773. The
 10 strain AF 2001 is a Gram-negative rod sized 0.4 - 0.6 x 1.0
 - 1.3 μ m and motile by means of a polar flagella. It is
 oxidative and cytochrome positive. These characteristics
 serve to identify the strain as a *Pseudomonas*. A summary of
 the key characteristics and the carbon utilization pattern
 15 for the growth is shown in Table 1 below. The strain AF
 2001 has similar characteristics to *Pseudomonas cepacia*
 disclosed in Bergy's manual of systematic bacteriology,
 1984. However, the strain AF 2001 is different from
Pseudomonas cepacia with regard to maltose, sucrose and
 20 adonitol utilization. It can grow on maltose and sucrose
 as a sole carbon source but can not grow on adonitol. In
 these aspects, the strain AF 2001 is thought to be a new
Pseudomonas cepacia.

25 Table 1. Characteristics of *Pseudomonas cepacia* AF 2001

	Cell type	Short rod
	Gram stain	Negative
	Biochemical characteristics:	
	Oxidative	Positive
30	Oxidase	Positive
	Catalase	Positive
	Urease	Negative
	DNase	Negative
	Lysine decarboxylase	Negative
35	Nitrate reduction	Negative
	Poly β -hydroxybutyrate accumulation	Positive

01 -contd.-

	Fluorescence	Negative
	Diffusible pigment	Positive
05	Esculin hydrolysis	Positive
	Benzene ring cleavage	Ortho
	Growth at 4 °C	Negative
	Growth at 41 °C	Positive
	Growth at pH 3	Negative
10	Growth at pH 9	Positive

Carbon utilization:

- Positive: maltose, sucrose, L-arabinose, mannose, xylose, fructose, tartarate, dulcitol, salicin, cellobiose, fumaric acid, caprate, threonine, lysine, arginine
- 15 Negative: adonitol, D-raffinose, lactose, maleic acid, inulin, ethylene glycol, phthalic acid, L-isoleucine, starch, melibiose

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Production of the Antibiotic

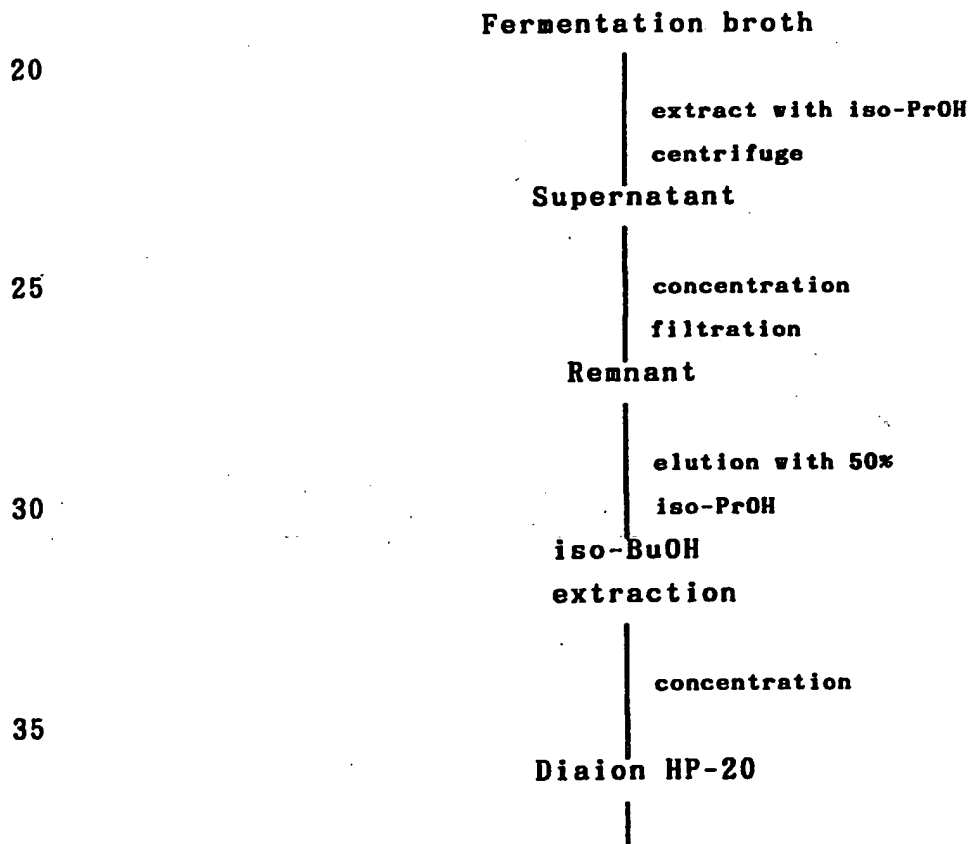
- Cultures of *Pseudomonas cepacia* AF 2001 were maintained at -85°C. When needed, working stock cultures were prepared
- 25 on agar slants composed of glucose 3%, peptone 1.5% and agar 2%. The slants were incubated at 28°C for 24 hours and used to inoculate germinator flasks containing 100ml of medium in 500ml Erlenmeyer flasks. This medium consisted of bactopectone 1%, yeast extract 1% and glucose
- 30 3%. The germinator was incubated for 24 hours at 28°C on a rotary shaker at 300 rpm, and then used to inoculate (1%) the same medium (10 L). The fermentation was run for 72 hours at 28°C with an agitation rate of 300 rpm. Progress of the fermentation and the subsequent isolation steps
- 35 were monitored by paper-disc, agar diffusion assay with *Candida albicans* ATCC 38245 as the assay microorganism. Cepacidine A was isolated and purified by the procedure

01 outlined in the diagram below. The fermentation broth(10 L)
was mixed with an equal volume of isopropanol(10 L), and the
mixture was adjusted to pH 4.0 with concentrated HCl. The
mixture was centrifuged and the cell pellet was discarded.
05 The supernatant was then concentrated under reduced
pressure to remove isopropanol. The precipitate that
formed during the concentration process contained the
bioactivity, as shown by conventional agar diffusion
assay. The concentrate and accompanying precipitate were
10 stored at 4°C for 6 hours to allow the precipitation to
proceed to completion. The precipitate was collected by
filtration with diatomaceous earth, washed with water and
then eluted with 50% isopropanol in water. This solution
was concentrated in vaccum to remove isopropanol. After
15 extraction of this concentrate with isobutanol-methanol
(8:2), the solvent extract was then concentrated under
reduced pressure. The precipitate that formed during the
concentration was collected by centrifugation and
dissolved in 50% isopropanol adjusted to pH 10.0 with
20 NaOH. This solution was diluted with 10 volume distilled
water and applied to diaion HP-20 resin column
chromatography. After elution with 50% isopropanol, the
eluent was concentrated in vaccum to a small volume. The
precipitate formed during the concentration was dissolved
25 in 50% isopropanol and applied to alumina column
chromatography. The passthrough that contained the
bioactivity was concentrated to a small volume and stored
at 4°C for 24 hours to allow precipitation. After
washing with water, the precipitate was dissolved in 50%
30 isopropanol and applied on a preparative ODS-silica gel
column of Waters μ -Bondapak C18 (3x30cm, 10 μ m) and
developed with acetonitrile-water (6:4, pH 3.5). The
fraction containing anti-candidal activity was collected
and concentrated in vaccum to give residues of pure
35 Cepacidine A (100mg). Cepacidine A was further resolved
into two closely related components, i.e., A₁ and A₂ by
HPTLC on silica gel(Merck Silica gel 60 F₂₅₄), eluting with

01 isopropanol-conc. ammonia-water(4:2:1). Visualization of
the components was accomplished either by charring after
spraying with a solution containing 5% ammonium molybdate
and 0.1% ceric ammonium sulfate in 10% sulfuric acid or by
05 spraying conventional ninhydrin reagent. The R_f values of
A₁ and A₂ are 0.53 and 0.58, respectively. The producing
ratio of A₁ and A₂ was determined to be 9:1 according to the
quantitative analysis of the amounts of purified A₁ and
A₂. Unfortunately, it was very difficult to isolate a
10 large amount of pure A₁ and A₂, because Cepacidine A was
not separated into A₁ and A₂ using the HPLC system
described above. Therefore, the mixture of A₁ and A₂ was
used for the following studies, *i.e.*, biological activity,
physicochemical properties and structural elucidation.

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Diagram on
Isolation Procedures of Cepacidine A₁ and A₂



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Biological activity

The *in vitro* antifungal activities of Cepacidine A and amphotericin B against a broad range of medically important fungi are listed in Table 2. The activities were determined by the twofold agar dilution method on potato dextrose agar medium. Cepacidine A exhibited a broad antifungal spectrum against all strains tested. Particularly, Cepacidine A was highly active against dermatophytes, namely *Microsporum canis*, *Trichophyton spp.*, and *Epidermophyton spp.*, and true yeast at concentrations lower than 0.049 µg/ml. The activities of Cepacidine A were greater than those of amphotericin B in most strains. However, no antibacterial activity was detected (MIC > 100 µg/ml) when Cepacidine A was assayed

01 against the bacteria i.e., *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

05 Table 2. Antifungal spectrum of
Cepacidine A and Amphotericine-B

	organism	KCTC No [*]	MIC (µg/ml)	
			Cepacidine A ^{**}	Amphotericin-B
10	<i>Candida albicans</i>	1940	0.391	0.782
	<i>Candida albicans</i>	38245	0.391	0.782
	<i>Candida glabrata</i>	1714	0.013	0.196
	<i>Cryptococcus neoformans</i>	1197	0.025	0.098
	<i>Saccharomyces cerevisiae</i>	1213	0.049	0.098
15	<i>Aspergillus niger</i>	2119	0.098	0.196
	<i>Microsporum gypseum</i>	1252	0.196	0.196
	<i>Microsporum canis</i>	11621	0.025	0.391
	<i>Epidennophyton floccosum</i>	1246	0.049	3.125
	<i>Trichophyton mentagrophyte</i>	6085	0.049	3.125
20	<i>Trichophyton rubrum</i>	38484	0.049	0.782
	<i>Fusarium oxysporum</i>	6084	0.196	0.196
	<i>Rhizopus stolonifer</i>	6062	0.391	0.391

* : Korean Collection for Type Cultures

** : Two component mixture

Physico-chemical properties

30

The physico-chemical properties of Cepacidine A are summarized in Table 3 below. Cepacidine A was isolated as white powder. The melting point ranged between 210°C and 214°C. Cepacidine A is insoluble in ethyl acetate, hexane, ether and benzene, and is hardly soluble in water, methanol, ethanol, isopropanol, butanol and acetone, while it is soluble in DMSO, alkali aqueous solution and acidic

35

01 aqueous solution. A 50% aqueous solution of alcohol
increases solubility. Cepacidine A showed positive
color reactions to aniline and ninhydrin reagents. The Rf
value of Cepacidine A on silica gel TLC developed with
05 n-butanol-acetic acid-water(3:1:1) was 0.18. However,
the Rf values of Cepacidine A on silica gel TLC developed
with isopropanol-water-saturated aqueous ammonia(4:1:2)
were separated as 0.53 and 0.58 so that the compound with
the Rf value of 0.53 was named Cepacidine A₁, and that
10 with 0.58, Cepacidine A₂. Since Cepacidine A₁ and
Cepacidine A₂ were hardly obtained separately by
prep-HPLC, unfortunately, the mixture, Cepacidine A, was
used for all spectrometric analyses, except TLC and amino
acid analysis by HPLC. The mixture has a 9:1 ratio of
15 Cepacidine A₁ and Cepacidine A₂. The UV spectrum of
Cepacidine A dissolved in water showed two maximum
absorption peaks at 232 nm and 274 nm, and the spectrum in
DMSO showed only one peak at 278 nm. Cepacidine A is very
stable in an aqueous solution between pH 2 and pH 11, while
20 unstable in an aqueous solution above pH 11.5 so that
Cepacidine A loses antifungal activities readily. The
molecular formula of Cepacidine A₁ was determined to be
C₅₂H₈₅O₂₂N₁₁ by HRFAB-MS, ¹³C NMR and elemental analysis
(calcd : C 51.4, H 7.0, N 12.7, O 29.0; found : C 51.5, H
25 8.0, N 11.0, O 29.5), Cepacidine A₂, C₅₂H₈₅O₂₁N₁₁. The
molecular ions of Cepacidine A₁ and Cepacidine A₂ by
HRFAB-MS were shown at m/z 1216.5999(MH⁺, calcd :
1216.5949) and 1200.5978(MH⁺, calcd. : 1200.5999),
respectively.

30

35

Table 3 Physico-Chemical Properties of Cepacidine A

	Cepacidine A ₁	Cepacidine A ₂
appearance	white powders	white powders
MP	210 - 214 °C	210 - 214 °C
UV λ _{max} nm (log ε) in H ₂ O	232 (2.8), 274 (1.7)	232 (2.8), 274 (1.7)
IR (KBr) ν _{max} cm ⁻¹	278 (1.2)	278 (1.2)
IR (KBr) ν _{max} cm ⁻¹	3352, 2924, 2854, 1666, 1539	3352, 2924, 2854, 1666, 1539
IR (KBr) ν _{max} cm ⁻¹	1412, 1252, 1069, 557	1412, 1252, 1069, 557
[α] _D ²⁵ H ₂ O	+ 20.8	+ 20.8
TLC R _f value (n-BuOH : AcOH : H ₂ O = 3 : 1 : 1)	0.18	0.18
(iso-PrOH : H ₂ O : satd NH ₄ OH = 4 : 1 : 2)	0.53	0.58
molecular formula	C ₅₂ H ₈₅ O ₂₂ N ₁₁	C ₅₂ H ₈₅ O ₂₁ N ₁₁
HRFA B-MS (M+H) ⁺ calcd	1216.5949	1200.5999
found	1216.5999	1200.5978

01 **Structure elucidation**

Since Cepacidine A showed positive color reaction to ninhydrin reagent, amino acid analysis was carried out by
05 TLC and HPLC after acid hydrolysis. The analysis revealed Cepacidine A₁ consists of β -hydroxy Asx, Asx, Ser, Gly and 2,4-diaminobutyric acid(1:1:2:1:1), and Cepacidine A₂, Asx, Ser, Gly and 2,4-diaminobutyric acid(2:2:1:1). For amino acid analysis, the solution obtained from acid
10 hydrolysis was eluted through octadecyl column. The remnant inside the column was washed with 50% isopropanol and collected for NMR experiments. The inspection of NMR experiments revealed the remnant is a type of β -amino acid with 18 carbons. This amino acid is a long chain of
15 carbons with 3 functional groups and 1 methyl group, which were determined to be 1 primary amine and 2 hydroxyl groups by ¹H NMR, ¹³C NMR, COSY, HETCOR, NOESY, HOHAHA and HMBC. Because Cepacidine A showed positive color reaction to aniline reagent, saccharide analysis was carried out by
20 cellulose TLC and HPLC after acid hydrolysis. The analysis revealed both Cepacidine A₁ and Cepacidine A₂ include xylose.

25 The spectrum of Cepacidine A obtained from low resolution FAB-MS shows only MH^+ ions of Cepacidine A₁ (m/z 1216) and Cepacidine A₂ (m/z 1200) except a few small fragments and xylose fragment. This phenomenon suggests Cepacidine A can be a cyclic peptide. The NMR experiments such as NOESY, HOHAHA and HMBC clarified this suggestion.

30 The sum of the calculated number of carbons of components obtained from amino acid analysis and saccharide analysis, and the number of carbons of C18 long chain amino acid is only 43. However, the ^{13}C NMR spectrum gives 50 peaks.

35 Therefore, the presence of the other components can be considered. The ^1H NMR spectrum of Cepacidine A reveals the presence of aromatic ring. Since the four carbon

01 signals at 114.70, 137.00, 132.20 and 156.50 ppm are
characteristic peaks caused by para-hydroxy phenyl group,
the presence of Tyr can be expected, but amino acid
analysis with HPLC does not show the peak of Tyr so that
05 the presence of a derivative of Tyr can be considered. In
the COSY spectrum, cross peaks among 4.19, 5.06, 6.67 and
7.14 ppm are observed. In addition, HETCOR shows four
correlated peaks such as 4.19 / 60.40, 5.06 / 70.96, 6.67 /
114.70 and 7.14 / 127.00 (^1H NMR / ^{13}C NMR). These phenomena
10 suggest one of β -protons of Tyr is substituted with a
hydroxyl group. In order to clarify this, a chemical
experiment was carried out. The UV spectrum of Cepacidine
A in DMSO (Fig. 1) shows one peak at 278 nm. An addition of
TFA into Cepacidine A caused bathochromic shift of λ_{max} to
15 312 nm. An elimination of α -proton and β -proton of Tyr can
cause a conjugation. Then, two carbon peaks at 114.70 and
127.00 in the ^{13}C NMR spectrum must be ϵ and δ carbons of
Tyr and denote two carbon intensity each. As a result, the
number of carbon in Cepacidine A is not 50 shown in the ^{13}C
20 NMR spectrum but 52.

As mentioned before, molecular formula of Cepacidine A₁ and
Cepacidine A₂ was determined to be C₅₂H₈₅O₂₂N₁₁ and C₅₂H₈₅O₂₁N₁₁
by HRFAB-MS and elemental analysis. In order to determine
25 Asx, pyrolyzed GC was carried out. Chromatograms of Asn
and Asp as references are shown in Fig. 6. Two
chromatograms can be distinguished by the characteristic
peaks at retention time = 47 min. Since the peaks of
Cepacidine A at the same retention time are same as those
30 of Asp, Asx contained in Cepacidine A must be Asp. NOESY
and HOHAHA experiments have revealed xylose is connected to
one of hydroxyl groups of C18 long chain amino acid. Here,
if one counts all elements contained in the determined
components of Cepacidine A₁, only β -hydroxy Asx is not
35 counted. Until now Gly (1), Ser (2), Asp (1), β -hydroxy
Tyr (1), 2,4-diaminobutyric acid (1), xylose (1), and C18
amino acid (C₁₈H₃₃O₄N₂) were determined and the sum of

01 elements contained in those components are $C_{48}H_{95}O_{27}N_9$.
 However, because Cepacidine A is a cyclic peptide and
 xylose is connected to C18 amino acid, the formula must be
 $C_{48}H_{79}O_{19}N_9$. The difference between this formula and that
 05 obtained from HRFAB-MS gives $C_4H_6O_3N_2$ for Cepacidine A_1 .
 Therefore, the undetermined components, β -hydroxy Asx of
 Cepacidine A_1 must be β -hydroxy Asn, and Asx of Cepacidine
 A_2 , Asn, respectively. The components of Cepacidine A_1 and
 Cepacidine A_2 are listed in Table 4.

10

Table 4. Components of Cepacidine A_1 and A_2

components	Cepacidine A_1	Cepacidine A_2
Glycine	1	2
15 Serine	2	2
Aspartic acid	1	1
Asparagine	0	1
β -Hydroxyasparagine	1	1
2,4-Diaminobutyric acid	1	1
20 β -Hydroxytyrosine	1	1
C ₁₈ Amino acid	1	1
Xylose	1	1

25

Experimental

FAB-MS was measured on a Jeol DX 303 spectrometer. UV and
 IR were recorded on a Beckman DU-70, and on a Bruker IFS 66,
 respectively. NMR spectra were recorded on a Bruker ARX
 30 400 spectrometer in DMSO- d_6 and 95% DMSO- d_6 / 5% D₂O. The
 elemental analysis data were obtained on Foss Heraeus
 CHN-O-Rapid. TLC was performed on pre-coated Silica gel
 plates(Merck catalog No. 5642). Pyrolysed GC was measured
 on a Shimadzu GC 15A and a JHP-35 Pyrolyser with a CBP-5
 35 column. For amino acid analysis Cepacidine A was
 hydrolyzed with 6N HCl at 105°C for 8 hr, and Waters amino
 acid conversion kit and Waters amino acid analysis column

01 were used, Waters Fluorescence 420, as a detector. For
saccharide analysis; Cepacidine A was hydrolyzed with 10%
H₂SO₄ at 100°C for 1 hr, and Waters carbohydrate analysis
system and Waters carbohydrate column were used, Waters
05 RI410, as a detector.

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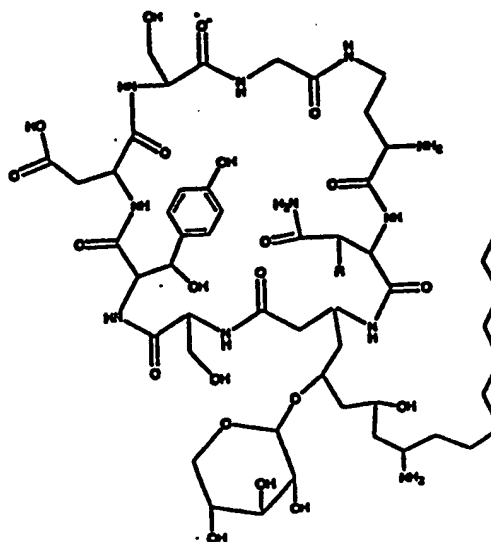
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01 What is claimed is :

1. An antifungal compound having the following formula:



wherein R is H or OH.

20 2. A microorganism *Pseudomonas* sp. AF 2001 producing the antifungal compounds according to claim 1.

25 3. A process for producing the antifungal antibiotics according to claim 1 which process comprises extracting and purifying the fermentation broth of the strain according to claim 2 to obtain the said antibiotics.

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Fig. 1 UV Spectrum of Cepacidine A

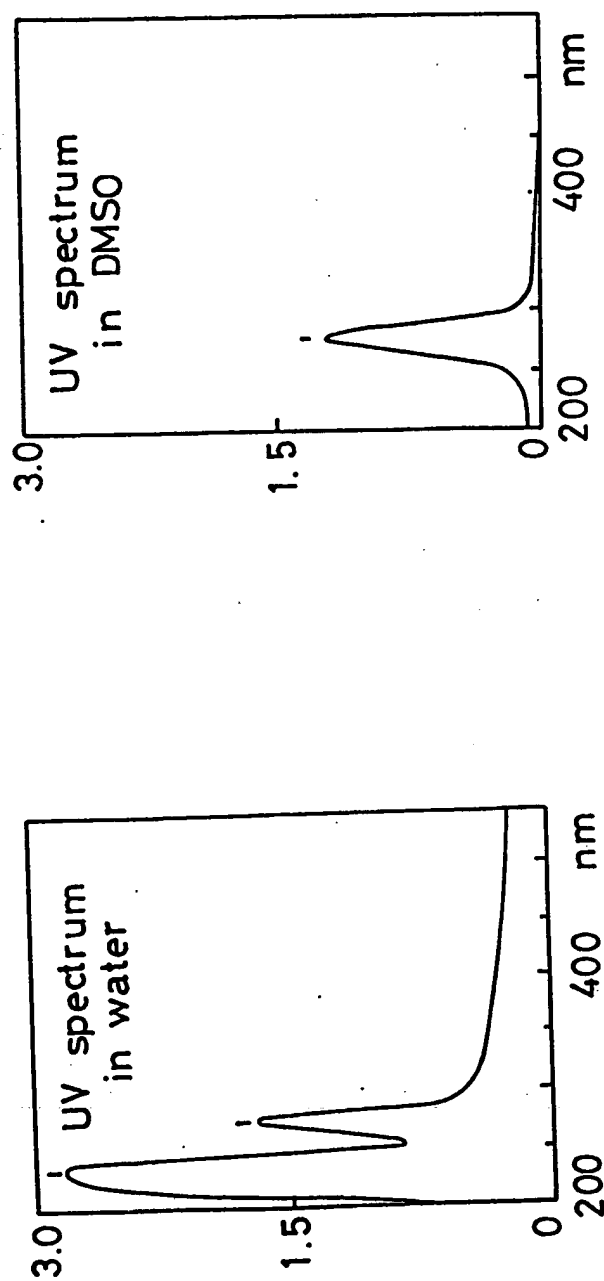


Fig. 2 IR Spectrum of Cepacidine A (in KBr)

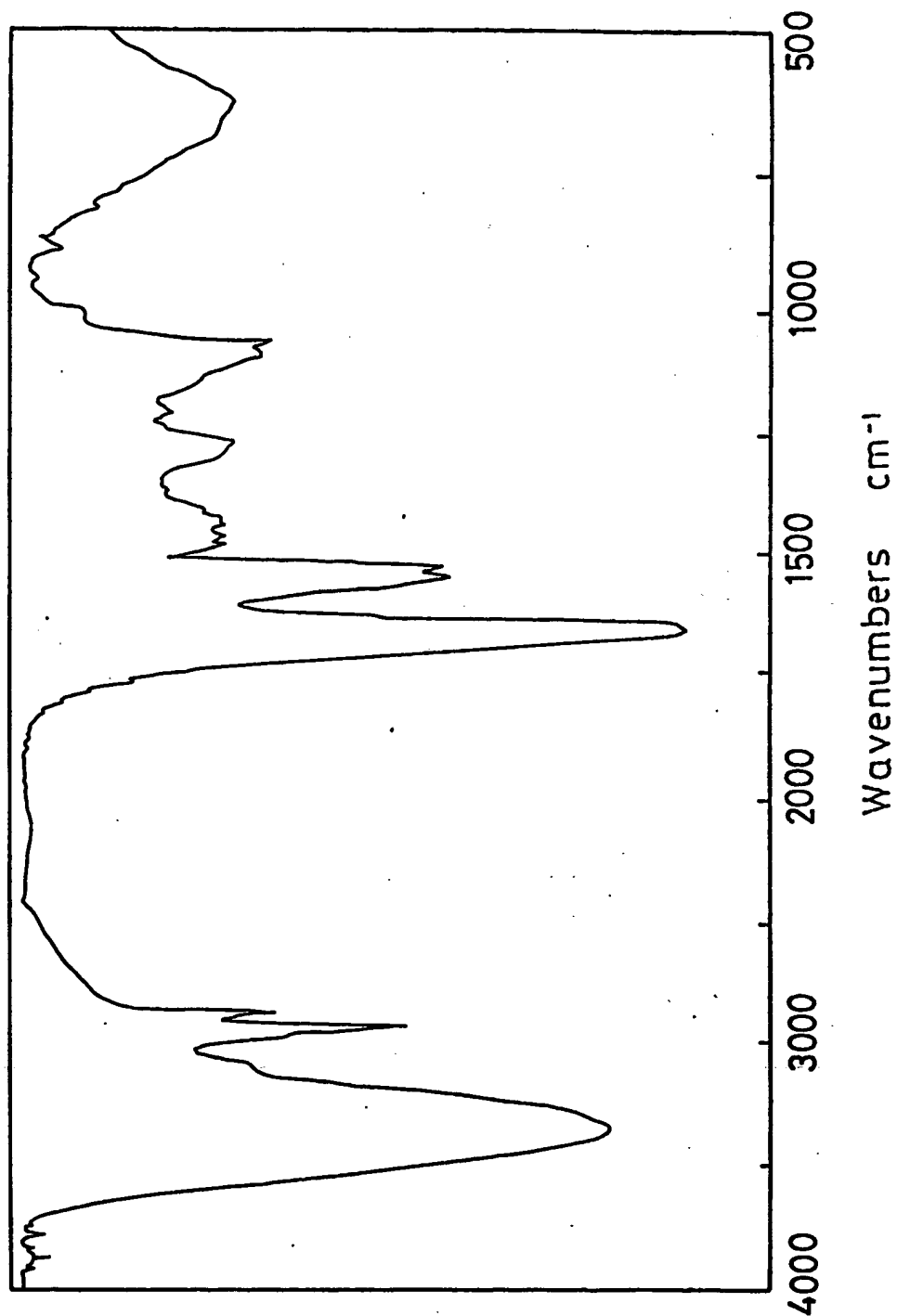
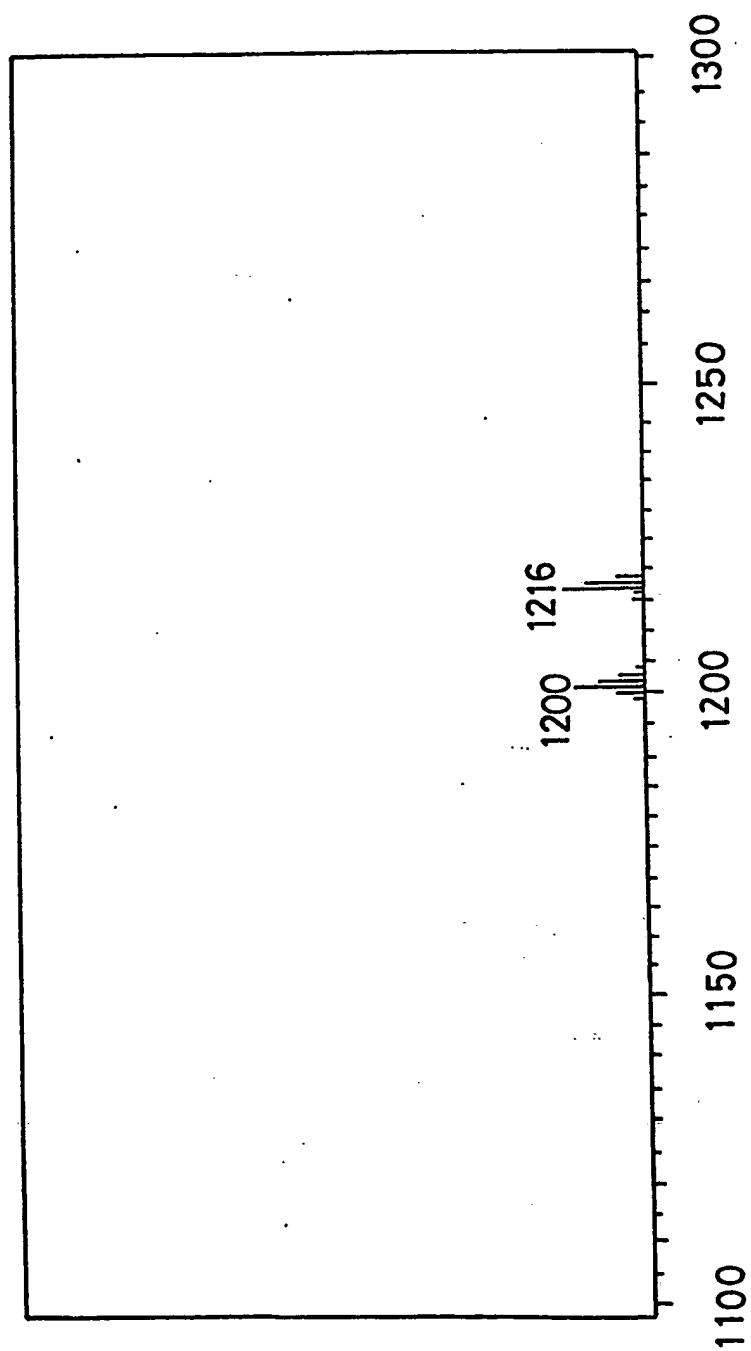


Fig. 3 FAB/MS Spectrum of Cepacidine A



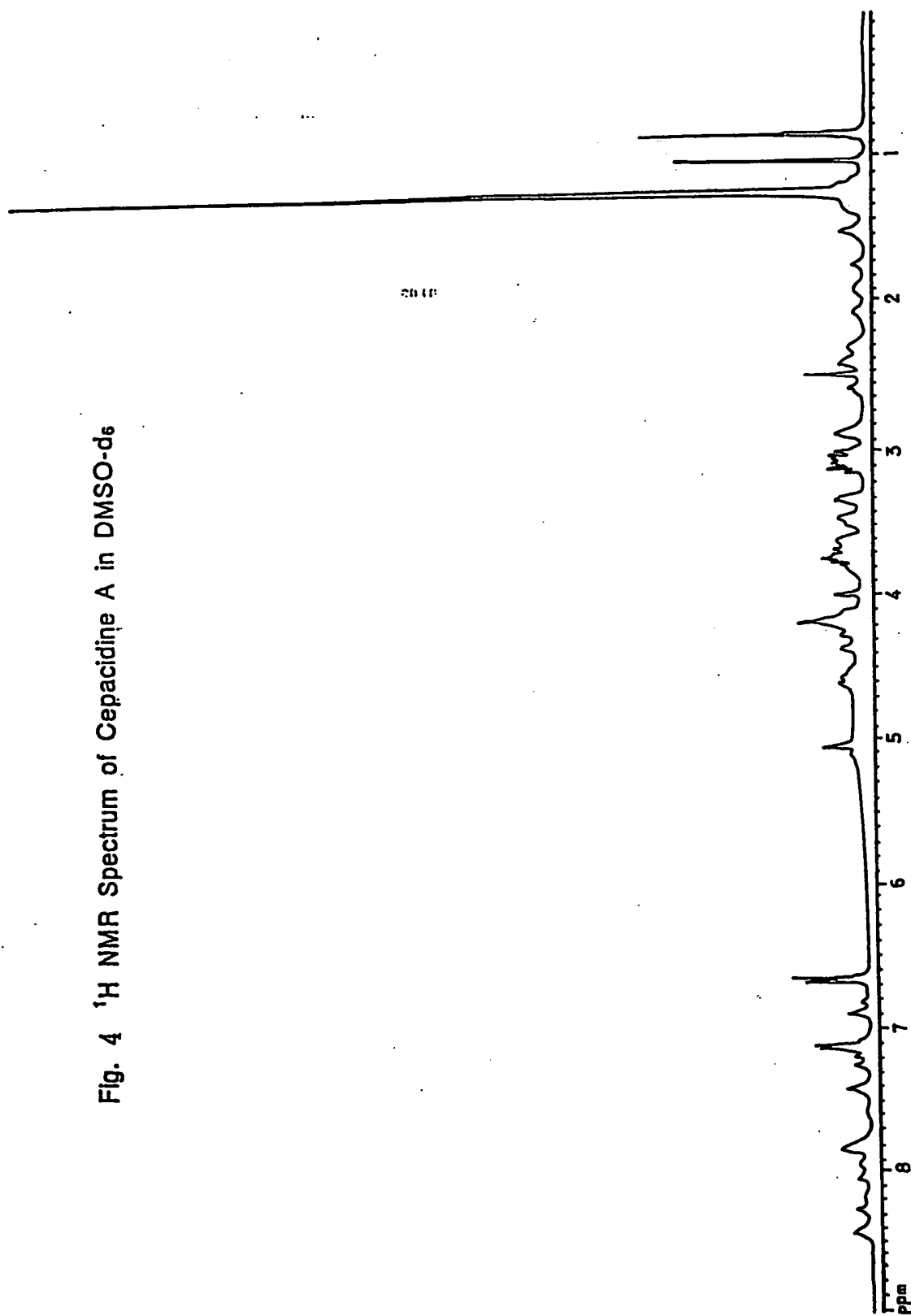


Fig. 4 ^1H NMR Spectrum of Cepacidine A in DMSO-d_6

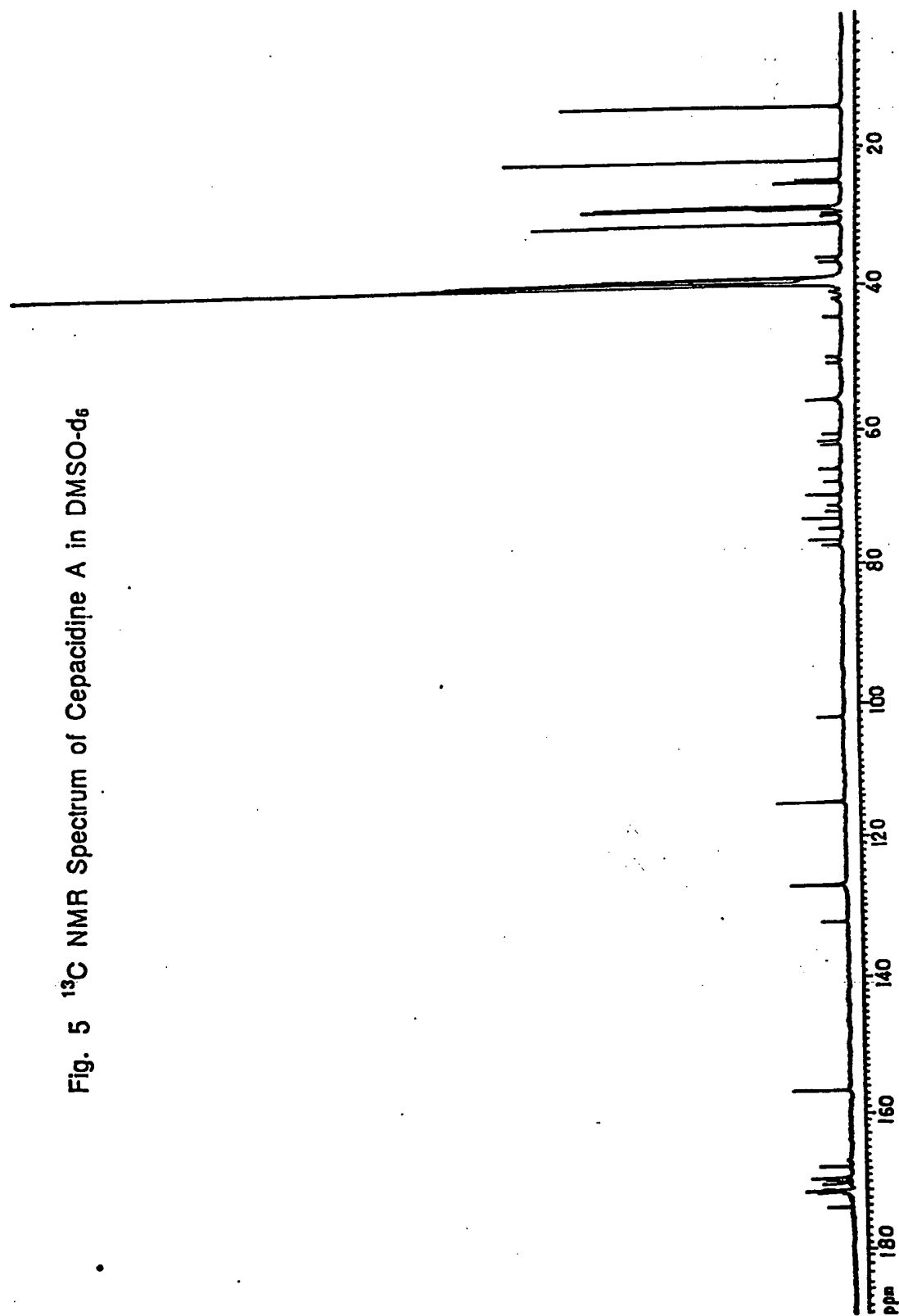
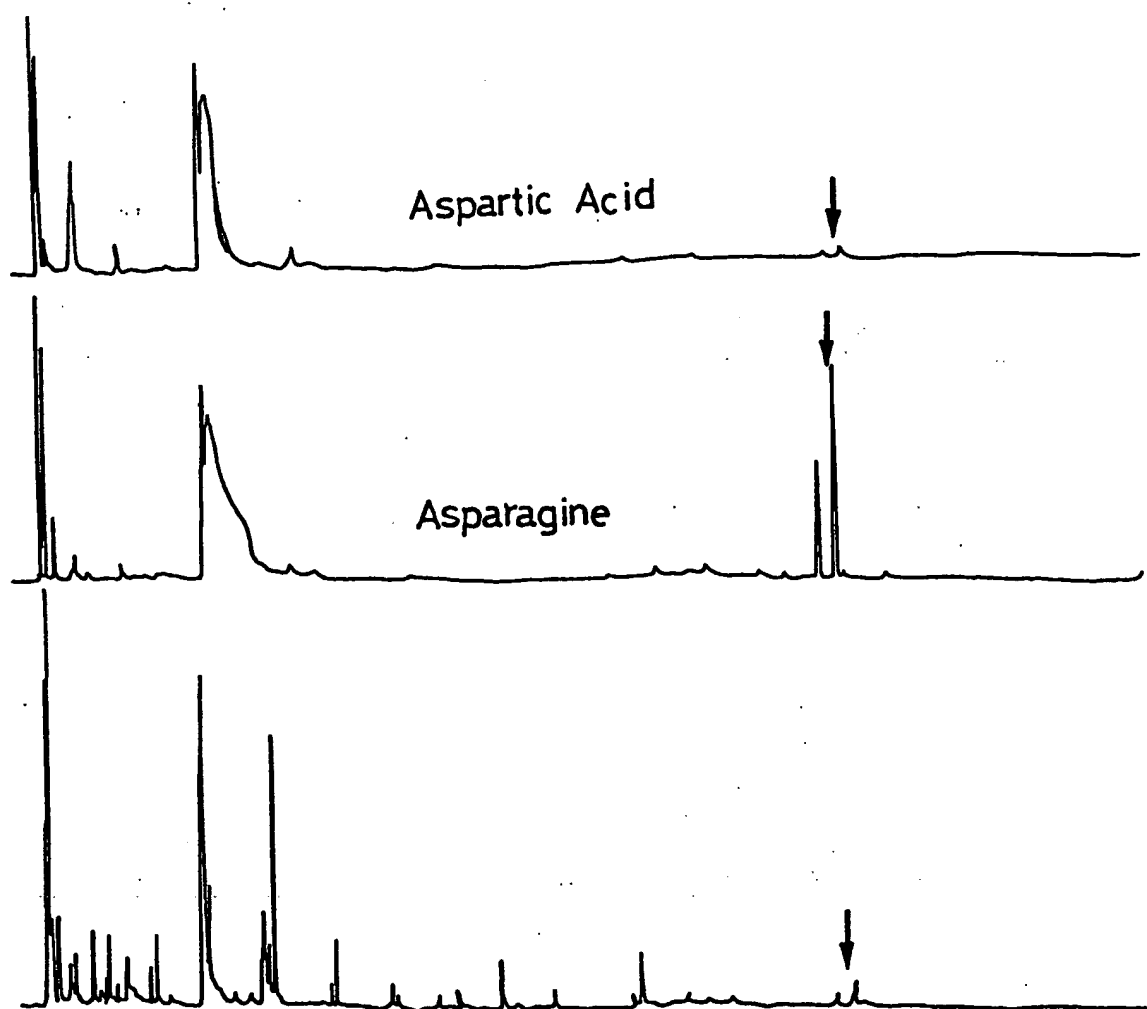


Fig. 5 ^{13}C NMR Spectrum of Cepacidine A in DMSO-d_6

Fig. 6 The Pyrolyzed Gas Chromatograms of Asp(top) , Asn(middle) and Cepacidine A(bottom)



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 94/00139

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 P 19/18, 1/04; C 12 N 1/20 // (C 12 N 1/20; C 12 R 1:38)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 P 19/18, 1/04; C 12 N 1/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4 601 904 (MEYERS et al.) 22 July 1986 (22.07.86), abstract. -----	1-3

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

27 December 1994 (27.12.94)

Date of mailing of the international search report

11 January 1995 (11.01.95)

Name and mailing address of the ISA/ AT

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/KR 94/00139

In Recherchenbericht angeführtes Patentedokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
US A 4601904	22-07-86	keine - none - rien	